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Genome-Wide Meta-Analyses of FTND and TTFC Phenotypes

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1 ABSTRACT

INTRODUCTION: FTND (Fagerström test for nicotine dependence) and TTFC (time to smoke first cigarette in the morning) are common measures of nicotine dependence (ND). However, genome-wide meta-analysis for these phenotypes has not been reported.

METHODS: Genome-wide meta-analyses for FTND (N = 19,431) and TTFC (N = 18,567) phenotypes were conducted for adult smokers of European ancestry from 14 independent cohorts.

RESULTS: We found that *SORBS2* on 4q35 ($P = 4.05 \times 10^{-8}$), BG182718 on 11q22 ($P = 1.02 \times 10^{-8}$), and AA333164 on 14q21 ($P = 4.11 \times 10^{-9}$) were associated with TTFC phenotype. We attempted replication of leading candidates with independent samples (FTND, N = 7,010 and TTFC, N = 10,061), however, due to limited power of the replication samples, the replication of these new loci did not reach significance. In gene-based analyses, *COPB2* was found associated with FTND phenotype, and *TFCP2L1*, *RELN* and *INO80C* were associated with TTFC phenotype. In pathway and network analyses, we found that the interconnected interactions among the endocytosis, regulation of actin cytoskeleton, axon guidance, MAPK signaling and chemokine signaling pathways were involved in ND.

CONCLUSIONS: Our analyses identified several promising candidates for both FTND and TTFC phenotypes, further verification of these candidates was necessary. Candidates supported by both FTND and TTFC (*CHRNA4*, *THSD7B*, *RBFOX1* and *ZNF804A*) were associated with addiction to alcohol, cocaine and heroin, and were associated with autism and schizophrenia. We also identified novel pathways involved in cigarette smoking. The pathway interactions highlighted the importance of receptor recycling and internalization in ND.

IMPLICATIONS: Understanding the genetic architecture of cigarette smoking and ND is critical to develop effective prevention and treatment. Our study identified novel candidates and biological pathways involved in FTND and TTFC phenotypes, this will facilitate further investigation of these candidates and pathways.

2 INTRODUCTION

Cigarette smoking imposes a high health and financial toll on the smokers as well as society at large. Regular cigarette smoking often leads to nicotine dependence (ND). The tendency to develop ND is influenced by both genetic predisposition and environmental factors. In recent years, genetic studies of ND have made significant progress, exemplified by the identification of the *CHRNA5-CHRNA3-CHRNA4*, *CHRNA3-CHRNA4*, *CHRNA4*, and *CYP2A6* loci.^{1–6} However, variants identified in these genes explain only a small proportion of heritability. For example, cigarettes smoked per day (CPD), a common measure used in ND studies, explained about 5.6% of the heritability.⁷ Many more risk genes remain unidentified.

Cigarette smoking is a complex behavior, and different measures are used to assess ND in both clinical and research settings. The quantity of consumption, typically assessed by self-reported CPD, is a measure used in many studies, including those that identified the *CHRNA5-CHRNA3-CHRNA4*, *CHRNA3-CHRNA4*, and *CYP2A6* loci.^{2–4} The Fagerström Test for Nicotine Dependence (FTND)⁸ is another commonly used measure in genetic studies. CPD is one of the six questions included in the FTND, and accounts up to 3 points in the 10-point FTND scale. Another FTND question, “How soon after you wake up do you smoke your first cigarette?” or time to smoke first cigarette in the morning (TTFC), is a strong predictor of difficulty to quit smoking and smoking relapse,^{9,10} COPD¹¹ and lung cancer.¹² While both FTND and TTFC are important measures of ND, **comparing to CPD**, only a few genome-wide association studies (GWASs)^{6,13–15} have been conducted using these phenotypes. ***CHRNA4* and a few intergenic loci were found to be associated with FTND,^{6,13} but no locus for TTFC was identified.**

Here we report results from our GWAS meta-analyses on FTND (N = 19,431) and TTFC (N = 18,567). Our motivation to analyze TTFC separately is to understand what genetic factors contributing to the difficulty of quitting and relapse. Another benefit is that we can compare the results between FTND and TTFC phenotypes and identify convergent candidates. A lesson learned from comprehensive studies of the *CHRNA5-CHRNA3-CHRNA4* locus is that a true signal can be detected with many correlated phenotypes.¹⁶ Hence, a candidate with corroborative support from both FTND and TTFC would be more credible.

3 METHODS

Datasets: We assembled 14 independent cohorts (FTND, N = 19,431; TTFC, N = 18,567) to examine the association between FTND/TTFC phenotypes and genome-wide genetic variants. A description of the cohorts and the summary of descriptive statistics of the cohorts is provided in the supplementary information file and Supplementary Table S1 respectively.

Genotype quality control and imputation: Genotype quality controls were conducted separately by individual groups for the discovery datasets ISIB, ALSPAC, AUTW, CEDAR, FTC1, FTC2, NTR, and GCD. For discovery datasets obtained from dbGaP (<http://www.ncbi.nlm.nih.gov/gap>) (SAGE, SC, MGS, COPDGene1, CIDR370v1, CIDR370v3, CIDRc3, CIDRc4 and EAGLE), we used the genotypes provided by the original investigators who conducted quality control procedures following the dbGaP standards. Genotype imputations were also conducted separately by each group using MaCH^{17,18} or IMPUTE2^{19,20} with the 1000 Genomes reference haplotypes (EUR panel, March 2012 release), using the default settings of the programs. Similarly, genotype quality control for the replication datasets (VTSABD, COPDGene2, S4S, PNAT2, and EAGLE [used for TTFC only]) was conducted by each group separately.

Inclusion criteria and phenotypes: Regular smokers, defined as those who smoked daily for at least one month or those who smoked at least 100 cigarettes lifetime, of European ancestry were included. FTND scores (0 – 10) and TTFC scores (0 – 3) were obtained from self-reported FTND questionnaire, ascertaining the smoking behaviors during the heaviest smoking period of the smokers. Both FTND and TTFC scores were treated as quantitative traits without transformation.

Association and meta-analyses: GWAS analyses were performed separately for each dataset by individual groups using the PLINK program.²¹ Assuming a linear **mixed effect** model, FTND and TTFC were treated as **continuous** outcomes and genotypes as predictors, while sex, age, and the first 10 principal components were included as covariates. Summary statistics from each dataset were combined by GWAMA²² program using inverse variance-weighted meta-analysis approach with fixed effects. **Because the individual samples were analyzed using the**

same model, the summary statistics were used directly without further normalization. For the meta-analyses, only bi-allelic single nucleotide polymorphisms (SNPs) with minor allele frequency $\geq 1\%$ and with high imputation quality (INFO value ≥ 0.4 from IMPUTE2, or $r^2 \geq 0.4$ from MaCH) were included. P-values from the meta-analyses were corrected for genomic-control. P-values below 5×10^{-8} were considered as genome-wide significant, while P-values below 5×10^{-5} were considered as suggestive association and this threshold was used for selection of loci for convergence analysis.

SNP heritability analyses: To estimate the SNP heritability with genome-wide data, we used the linkage disequilibrium (LD) adjusted kinship algorithm.^{23,24} Specifically, we used the control subjects from two large studies^{25,26} as reference to select LD adjusted SNP predictors, and used the meta-analysis results from the FTND and TTFC to estimate the weights for these SNPs. The heritability was then estimated with these LD tagged SNPs. The number of SNPs used for FTND heritability estimate was 2, 857,113 and that for TTFC was 2, 981,471.

Replication analyses: Six independent datasets of European descent (N = 7,010 for FTND, and N=10,061 for TTFC) were used for a replication study of selected SNPs. We conducted replication study for all loci meeting these four criteria: (i) having five or more SNPs with P-value $\leq 5 \times 10^{-5}$; (ii) at least one SNP with P-value $\leq 5 \times 10^{-5}$ having a minor allele frequency $\geq 5\%$; (iii) minor allele frequency variation at the locus $> 5\%$ between SNPs with P-value $\leq 5 \times 10^{-5}$; and (iv) at least four datasets contributed to the signal at the locus. For each locus, we selected the SNP with smallest P-value for replication testing. The selection of these criteria were based on lessons learned from recent GWASs where true loci have multiple associated SNPs with different frequencies and many loci have multiple independent signals.^{16,27} The requirement of 5% minor allele frequency was intended to minimize the influence of potential outlier SNPs, and the inclusion of variation of minor allele frequency was to maximize the likelihood that the locus could harbor more than one association signal. P-values below 0.0031 (0.05/16) were considered as significant replication.

Gene-based association analyses: We performed gene-based association analyses using the results from the GWAS meta-analyses. Specifically, we used the Knowledge-based mining system for Genome-wide Genetic studies (KGG) software,^{28,29} which uses an extended Simes

test to integrate functional information and association evidence to combine the SNP P-values within a gene to obtain an overall P-value for each gene. In these analyses, we filtered out SNPs found in less than four datasets. All analyses were conducted using KGG default settings. We used the Benjamini-Hochberg method³⁰ to correct for multiple testing, and considered FDR q-values below 0.05 as statistically significant.

Pathway and network analyses: We conducted pathway enrichment analysis for genes with at least one marker with $P \leq 5 \times 10^{-5}$ from GWAS meta-analyses of either FTND or TTFC. If a marker was within a gene region, it was assigned to the gene; otherwise, it was mapped to its most proximate gene using the 50kb flanking regions (both 5' and 3' sides). Genes identified using SNPs associated with FTND and TTFC were merged for the pathway enrichment analyses. We used the hypergeometric test implemented in the tool WebGestalt (2014 update)^{31,32} and the canonical pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. We required each pathway to have at least three genes from our gene list and no more than 300 genes from the reference genome. The P-values from hypergeometric tests were further adjusted by the Benjamini-Hochberg method.³⁰ Only pathways with adjusted P-values < 0.05 were considered statistically significantly enriched.

We further examined how enriched pathways interacted with each other in function and regulation. Specifically, we applied the Characteristic Sub-Pathway Network (CSPN) algorithm³³ to search for significantly interacting pathway pairs.³⁴ CSPN was designed to prioritize pathway pairs with significant interaction of molecules from each pathway pair in the human protein-protein interaction (PPI) network (details in reference³³). We used the human PPI data from the Protein Interaction Network Analysis (PINA) platform³⁵ as the reference network in this pathway crosstalk analysis. Our working PPI network included a total of 11,318 nodes (protein-coding genes) and 67,936 interactions. We restricted the analysis specifically to the aforementioned merged gene set and their enriched pathways. When running CSPN, a mode "OR" was selected, i.e. we considered all PPIs formed by the supplied genes as well as their one-step extension. In the final step, we selected the significant pathway interaction pairs based on permutation P-values ≤ 0.05 .

4 RESULTS

4.1 *FTND and TTFC GWAS meta-analyses*

In the GWAS meta-analyses of the discovery sample, about 19,000 subjects from 14 independent cohorts were included. For FTND (N = 19,431), only the *CHRNA5-CHRNA3-CHRNA4* locus reached genome-wide significance (Table 1). The smallest P-value was observed at rs16969968 (Beta = -0.21, SE = 0.02, P = 3.96×10^{-19}), which is a functional variant in *CHRNA5* known to influence smoking behavior, in particular the quantity of cigarettes smoked. Several additional loci (*CIB4* on 2p23, BG182718 on 11q22, and *DSC3* on 18q12) were promising (Table 1). The meta-analysis had a lambda of 1.03, indicating no significant genomic inflation. The Manhattan and Q-Q plots for FTND are shown in Figure S1. In the analyses of TTFC (N = 18,567), four loci (*SORBS2* on 4q35, BG182718 on 11q22, AA333164 on 14q21, and *CHRNA5-CHRNA3-CHRNA4* on 15q25) reached genome-wide significance (Table 1). A closer examination of the local linkage disequilibrium (LD) indicated that the signals from BG182718 and *CHRNA5-CHRNA3-CHRNA4* covered a broad genomic region (~500 kb) while the signals from *SORBS2* and AA333164 were restricted within small intervals (Figure 1). *CHRNA3-CHRNA6* (smallest P = 8.83×10^{-8} for rs11785369) and *MIR31HG* (smallest P = 5.70×10^{-8} for rs184042824) were approaching genome-wide significance. Several loci meeting our criteria for replication testing were also listed in Table 1. No genomic inflation was observed for TTFC phenotype (lambda=1.01). The Manhattan and Q-Q plots for TTFC are shown in Figure S1.

For FTND, four loci were selected for replication in several independent samples (Table 2). Of the four loci tested, only rs16969968 at the *CHRNA5-CHRNA3-CHRNA4* locus was successfully replicated (P = 1.19×10^{-11}). Rs17005545 in the novel locus *CIB4* had a P-value of 2.68×10^{-7} in the combined samples. For TTFC, rs16969968 in *CHRNA5* and 11 other loci were selected for replication. Rrs16969968 in *CHRNA5* (P = 2.84×10^{-10}) was successfully replicated and rs11785369 near the *CHRNA3* gene reached genome-wide significance in the combined samples (Table 2).

We conducted SNP heritability analyses for both FTND and TTFC using the LD adjusted kinship algorithm.^{23,24} The SNP heritability for FTND was estimated at 0.645, and that for TTFC was 0.193.

4.2 Convergent loci between FTND and TTFC

We compared the results obtained for FTND and TTFC to identify genes showing convergent association signals. We then selected genes/loci showing suggestive association ($P \leq 5 \times 10^{-5}$) in both FTND and TTFC meta-analyses. A total of 15 genes/loci with convergent association signals were found (Table 3). The *CHRNA5-CHRNA3-CHRNA4* locus was the only one reaching genome-wide significance for both FTND and TTFC. Other nicotinic receptors, *CHRNA3-CHRNA6* and *CHRNA4*, also showed convergent association signals. Other highlighted genes included long non-coding RNAs (DA409732 and BG182718) and a RNA binding gene (*RBFOX1*), microtubule and actin regulation genes (*KIF2B* and *VAV2*), a cAMP/cGMP modulating gene (*PDE2A*), and an apolipoprotein gene (*APOL3*). For some genes (*THSD7B*, *PDE2A*, *KIF2B*, and *APOL3*), the same SNPs showed suggestive association for both phenotypes; for other genes (*ZNF804A*, *VAV2*, *RBFOX1*, and *CHRNA4*), the association signals for the two phenotypes were from different SNPs.

4.3 Gene-based meta-analyses

We conducted gene-based analyses using the KGG program. In these analyses, in addition to the *CHRNA5* locus and nearby genes, a few novel genes were identified (Table 4). *COPB2* and *CHRNA4* reached significance for association with FTND, and *TTFCP2L1*, *RELN*, and *INO80C* were significant for the TTFC phenotype. Table 4 also lists other candidates from gene-based analyses (i.e., genes with q-value ≤ 0.1).

4.4 Pathway and network analyses

There were 647 SNPs with P -value $\leq 5 \times 10^{-5}$ in the FTND meta-analysis and they were mapped to 134 known genes. There were 936 markers with P -value $\leq 5 \times 10^{-5}$ in the TTFC meta-analysis and they were mapped to 145 genes. Altogether 15 genes were shared between the two phenotypes, yielding a total of 265 unique genes for pathway analyses. In the enrichment analyses using canonical pathways defined in KEGG database, a total of 26 pathways were

found to be overrepresented among these genes (Table 5). The neuroactive ligand-receptor interaction pathway was the most significantly enriched pathway, including nicotinic receptors (*CHRNA5*, *CHRNA3*, *CHRNA4*, *CHRNA6*, and *CHRNA4*), gamma-aminobutyric acid receptor (*GABRG3*), glutamate receptor (*GRM5*), and somatostatin receptors (*SSTR1* and *SSTR4*). In addition to several pathways known to be involved in ND (cell adhesion molecules, MAPK signaling, tight junction, and axon guidance), our analyses suggested that endocytosis, lysosome, chemokine signaling, and regulation of actin cytoskeleton pathways are also involved in ND.

We further tested if and how these pathways interacted with each other. Multiple immune related pathways were identified by two highlighted genes, *HLA-DMB* and *HLA-DRB5*; to avoid the extensive network of HLA related immune responses, we excluded these two genes in our pathway crosstalk analyses. Our analyses revealed interacting networks among the endocytosis, regulation of actin cytoskeleton, MAPK signaling, axon guidance, and chemokine signaling pathways (Figure 2). We also saw that two other pathways, melanogenesis and basal cell carcinoma, interacted and contributed to ND phenotypes.

5 CONCLUSION AND DISCUSSION

We conducted GWAS meta-analyses for the FTND and TTFC phenotypes in about 19,000 regular smokers of European ancestry from 14 independent cohorts. We confirmed the known association of the functional variant D398N (rs16969968) in the *CHRNA5-CHRNA3-CHRNA4* locus for both FTND and TTFC phenotypes. While the association between FTND and *CHRNA5-CHRNA3-CHRNA4* locus had been reported before, ours is the first to report its association with TTFC. GWAS meta-analysis of FTND identified one potential novel locus (*CIB4* on 2p23) with suggestive association in the discovery samples and a trend in the replication samples. *CIB4* encodes a calcium binding protein that interacts with integrin.³⁶ It may be involved in the integrin signaling. Our GWAS meta-analyses of TTFC, the largest for this phenotype, identified three novel loci (*SORBS2* on 4q35, BG182718 on 11q22, and AA333164 on 14q21) in the discovery samples. *SORBS2* encodes an adaptor protein involved in the regulation of actin cytoskeleton,³⁷ and is recently suggested to be involved in intellectual disability.³⁸ The 11q22

signal peaks at rs117029742 located 25 kb from the 3' end of BG182718 (also referred to as RP11-379J13.2). BG182718 is a long non-coding RNA with unknown function. In the interval of 1.5 MB centered at rs117029742, there are no other known genes. Interestingly, ClinVar database (<http://www.ncbi.nlm.nih.gov/clinvar/>) reports multiple large copy number variants in this region and these variants are reported to be associated with developmental disabilities.³⁹ AA333164 (also referred to as RP11-305B6.3) on 14q21 is another long non-coding RNA with unknown function; it also overlaps with known copy number variants associated with global developmental delay.³⁹ It is not clear how TTFC relates to intellectual disability or developmental delay, or whether there are other functions that account for the association. Independent replication of the three novel loci for TTFC was not evident, presumably due to the lack of power of the replication samples. Statistically significant evidence for independent replication of the observed SNP associations may require even larger sample sizes.

FTND is a commonly used measure in studies of smoking behavior and ND. It consists of six questions, with a sum score ranging from 0 to 10.⁸ Many studies have used FTND scores to define ND, with varying thresholds (e.g., $FTND \leq 3$ as low ND and > 6 as high ND, but also $FTND \geq 4$ as dependent and $FTND=0$ as unaffected and those in-between as uncertain), while others have treated FTND as a quantitative trait. Hancock and colleagues⁶ conducted a GWAS using categorized FTND (low-level smoking and mild ND [$FTND = 0$ to 3]; moderate ND [$FTND = 4$ to 6]; and severe ND [$FTND = 7$ to 10]) and discovered that *CHRNA4* was associated with FTND at genome-wide significance across GWAS discovery and replication samples. Loukola and colleagues¹⁴ used FTND as a binary trait ($FTND \geq 4$ as affected) in a study of 1,114 Finns but detected no genome-wide significant associations. Gelernter and colleagues¹³ conducted a GWAS with FTND as a quantitative trait in a sample of 4,117 Caucasians and 3,529 African Americans, and detected a genome-wide significant signal in an intergenic region on 7q21. In our analyses, which included the Caucasian subjects from the study of Gelernter and colleagues, only the *CHRNA5-CHRNA3-CHRNA4* locus reached genome-wide significance. When the discovery and replication samples were combined, the signal amplified. The signal observed in Gelernter et al study,¹³ rs13225753, was not significant in our analyses ($P = 0.7188$), presumably due to heterogeneity at the locus (Cochran's $Q = 36.86$, $P = 4.36 \times 10^{-4}$).

Compared to FTND, the TTFC phenotype is more related to the ability to quitting and relapse⁹. Loukola and colleagues¹⁴ conducted a GWAS of TTFC in 1,114 Finnish subjects, but detected no genome-wide significant signals. In our discovery sample, which also included the subjects from the study of Loukola and colleagues¹⁴, we found four loci reaching genome-wide significance (Table 1). When the discovery and replication samples were combined, the *CHRNA5-CHRNA3-CHRNA4* locus remained genome-wide significant for both phenotypes and the *CHRNA3-CHRNA6* locus reached genome-wide significance for TTFC, while the signals of other loci decreased somewhat due to weaker magnitudes of association in the replication samples (the common “winner’s curse” phenomenon⁴⁰). The *CHRNA3-CHRNA6* locus was originally identified using CPD phenotype,³ in the current study a much stronger signal was detected for TTFC (min $P = 8.83 \times 10^{-8}$ for rs11785396) compared to FTND (minimal $P = 2.28 \times 10^{-5}$ for rs11785396) (see Table 3). This is consistent with a previous report that the signal at the *CHRNA3-CHRNA6* locus is stronger when analyzed with FTND phenotype than that of CPD,¹⁵ because the main difference between FTND score and CPD is largely contributed by TTFC score.

TTFC is a component of FTND, and therefore TTFC scores correlate with FTND scores. In the literature, the correlation coefficient was reported to be 0.57.⁴¹ In the SC sample, the correlation coefficient was 0.69. However, when we examined the meta-analysis results from these phenotypes, we saw notable differences. For markers reaching genome-wide significance, the *CHRNA5-CHRNA3-CHRNA4* locus was the only region showing overlap. Even for this locus, the strength of signal differed substantially between FTND (minimal $P = 3.96 \times 10^{-19}$ for rs16969968) and TTFC (minimal $P = 6.21 \times 10^{-9}$ for rs16969968), despite similar effective sample sizes (Table 1). BG182718 (tagged by rs117029742) also showed signals for both phenotypes, with stronger association with TTFC ($P = 1.02 \times 10^{-8}$) compared to FTND ($P = 1.04 \times 10^{-7}$). On the other hand, there were genes/loci that showed association with one phenotype but not the other. For example, *CIB4* (tagged by rs17005545) showed suggestive association with FTND ($P = 6.99 \times 10^{-7}$), but very weak evidence of association with TTFC ($P = 2.15 \times 10^{-4}$). Similarly, rs34022242 located between *MFSD2A* and *CAP1* showed suggestive association with TTFC ($P = 2.48 \times 10^{-7}$), but no association with FTND ($P = 0.024$). This suggests that FTND and TTFC measure different aspects of ND. Based on this information, we reasoned that if some genes/loci showed

reasonable signals for both phenotypes, it could be seen as a convergent evidence that these genes/loci are likely genuinely associated with ND. Our convergent analyses identified several intriguing genes in addition to those previously highlighted for ND (*CHRNA5-CHRNA3-CHRNA4*, *CHRNA3-CHRNA6* and *CHRNA4*⁶). These included genes previously associated with diseases for which cigarette smoking is a significant risk factor, such as cocaine addiction (*RBFOX1*),⁴² alcohol dependence (*THSD7B*),⁴³ schizophrenia (*ZNF804A*)⁴⁴ and heroin addiction^{45,46} (*ZNF804A*), rheumatoid arthritis (*PDE2A*),⁴⁷ and prostate cancer (*APOL3*)⁴⁸ (Table 3). Furthermore, *RBFOX1* was highlighted in a recent study on the genetic relationship between schizophrenia and ND.⁴⁹ It remains to be elucidated whether these genes are independent risk factors for these diseases among non-smokers, or is the genetic association arising from mediation or moderation by smoking. For example the *CHRNA5* D398N function variant (rs16969968) is a risk factor for lung cancer but only among smokers.^{50,51} Our results further reassured that for a true signal, such as the *CHRNA5-CHRNA3-CHRNA4* locus, convergent signals are seen with multiple related phenotypes.

We also noticed the difference in SNP heritability estimates between the FTND and TTFC phenotypes. The SNP heritability estimate of 0.645 for FTND was close to the heritability estimated from twin studies for ND.⁵² The estimate of 0.193 for TTFC seemed low as compared to twin studies,⁵³ but it was close to the estimate of 0.154 from a study with SNP measure.⁵⁴ The implication of this difference was not immediately clear. As SNP heritability estimates were influenced by the power of the GWASs, it was likely that our TTFC meta-analysis did not have the power to have a good estimate of heritability.

Gene-based analyses highlighted several other genes besides the *CHRNA5-CHRNA3-CHRNA4* and *CHRNA4* loci, including a gene involved in pluripotency demethylation and development (*TTFCP2L1*),^{55,56} a gene associated with lung cancer (*COPB2*),⁵⁷ a gene implicated in schizophrenia (*RELN*),^{58,59} and a gene involved in chromatin remodeling (*INO80C*)⁶⁰ (Table 4). These were novel genes first reported to be associated with smoking related phenotypes. It would be interesting to see whether these genes could be replicated in future studies. Our pathway analyses revealed two statistically significantly enriched pathways. While the involvement of neuroactive ligand-receptor interaction pathway in ND was expected since

nicotinic receptors and other surface receptors belong to this pathway, the identification of endocytosis pathway in ND was novel and interesting. Over the years, there has been accumulating evidence that the recycling and internalization of surface receptors, such as nicotinic receptors,⁶¹ NMDA receptors,⁶² glutamate receptors,⁶³ and opioid receptors,⁶⁴ are involved in drug addiction. Furthermore, it is known that the recycling and internalization of surface proteins are mediated and regulated by endocytosis, actin cytoskeleton, and lysosome functions. Our pathway analyses explicitly identified these pathways in ND (Table 5). Our network analyses indicated that these pathways were interacting with each other, forming an interconnected network (Figure 2). These findings were consistent with the emerging picture in addiction studies.

In summary, our GWAS meta-analyses of FTND and TTFC identified several promising candidates for both phenotypes. Three novel loci (*SORBS2*, BG182718, and AA333164) were discovered for TTFC. Although we could not validate these novel loci with statistical significance in our replication sample, further investigation is warranted. With supporting information from both FTND and TTFC phenotypes, we also identified promising candidates for ND, including several genes known for association with other psychiatric disorders. Our pathway analyses highlighted the endocytosis pathway, supporting the importance of recycling and internalization of surface receptors in the development of nicotine addiction. In our network analyses, we discovered a multi-node network with several interacting pathways known for involvement in substance abuse and psychiatric disorders. This information provides new insights for our understanding of ND and nicotine withdrawal.

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Table 1. The most significant loci identified in the FTND and TTFC GWAS meta-analyses in the discovery sample

	CHR	BP	SNP	EA	NEA	EAF	BETA	SE	Z	P-Value	N	Effect *	GENE
FTND	2	26,860,822	rs17005545	T	C	0.94	-0.23	0.05	-4.97	6.99E-07	18621	-----+-----	CIB4
	11	97,713,579	rs117029742	T	C	0.98	-0.64	0.12	-5.32	1.04E-07	12977	-----? ? - ? - + ?	BG182718
	15	78,882,925	rs16969968	G	A	0.64	-0.21	0.02	-8.83	3.96E-19	17860	-----? - + -	CHRNA5
	18	28,355,510	rs28510557	C	T	0.60	-0.10	0.02	-4.85	1.23E-06	18673	-----+ + - - - + - -	DSC3
TTFC	1	40,464,894	rs34022242	C	G	0.94	-0.13	0.02	-5.16	2.48E-07	14286	-----+ ? - - ? ?	MFSD2A
	1	68,862,622	rs3125927	G	A	0.64	-0.05	0.01	-5.02	5.24E-07	16091	-----+ - - - - ?	RPE65
	4	103,715,385	rs223441	C	T	0.51	0.05	0.01	4.58	4.66E-06	18533	++++-+++++ - + + +	CISD2
	4	186,514,078	rs28567706	C	G	0.99	0.49	0.09	5.49	4.05E-08	6533	+ ? ? ? ? ? + ? ? ? ? + ? ? ?	SORBS2
	8	42,527,386	rs11785369	A	C	0.89	0.09	0.02	5.35	8.83E-08	18634	+++++ + + + + - + - + +	CHRNA3
	9	21,673,859	rs184042824	C	T	0.98	-0.31	0.06	-5.43	5.70E-08	9005	- + - - ? + ? - - ? ? ? ? - ?	MIR31HG
	11	97,713,579	rs117029742	T	C	0.98	-0.30	0.05	-5.73	1.02E-08	13061	-----? + - ? + + ?	BG182718
	14	44,608,436	rs10133756	C	G	0.98	-0.31	0.05	-5.88	4.11E-09	13283	---? ? - - - - ? - ? ? + -	AA333164
	15	78,882,925	rs16969968	G	A	0.66	-0.06	0.01	-5.82	6.21E-09	17834	-----+ - ? + - -	CHRNA5
	17	51,940,280	rs2877510	C	T	0.17	0.06	0.01	4.52	6.36E-06	18647	++++- - + - - + - - + +	KIF2B
	20	15,901,883	rs6135601	G	T	0.97	-0.13	0.03	-4.55	5.53E-06	18644	-----+ - - - - + - - - -	MACROD2
	22	42,058,846	rs132786	G	A	0.19	-0.07	0.01	-4.61	4.07E-06	14605	--? - - - - - - ? - - - -	XRCC6

EA, effect allele; NEA, Non-effect allele; EAF, effect allele frequency; BP, base-pair position according to GRCh37/hg19; FTND, Fagerström Test for Nicotine Dependence; TTFC, time to first cigarette. *, minus and plus signs refer to the direction of effect in each independent dataset (SAGE, SC, MGS, COPDGene1, ISIB, CIDR370v1, CIDR370v3, CIDRc3, CIDRc4, EAGLE, ALSPAC, AUTW, VTSABD, CEDAR, FTC1, FTC2, NTR, GCD), while '?' refers to missing data. Genome-wide significant signals are highlighted in bold.

Table 2. Replication of candidates for FTND and TTFC

Marker Info						Discovery			Replication			Combined			
CHR	BP	SNP	EA	NEA	GENE	BETA	SE	P-value	BETA	SE	P-Value	BETA	SE	P-Value	
FTND	2	26,860,822	rs17005545	T	C	CIB4	-0.23	0.05	6.99E-07	-0.16	0.10	0.1078	-0.22	0.04	2.68E-07
	11	97,713,579	rs117029742	T	C	BG182718	-0.64	0.12	1.04E-07	0.29	0.19	0.1370	-0.38	0.10	1.73E-04
	15	78,882,925	rs16969968	G	A	CHRNA5	-0.21	0.02	3.96E-19	-0.29	0.04	1.19E-11	-0.23	0.02	4.24E-28
	18	28,355,510	rs28510557	C	T	DSC3	-0.10	0.02	1.23E-06	0.02	0.04	0.6672	-0.08	0.02	3.19E-05
TFC	1	40,464,894	rs34022242	C	G	MFSD2A	-0.13	0.02	2.48E-07	-0.05	0.04	0.1643	-0.11	0.02	3.53E-07
	1	68,862,622	rs3125927	G	A	RPE65	-0.05	0.01	5.24E-07	0.02	0.02	0.2298	-0.03	0.01	2.25E-04
	4	103,715,385	rs223441	C	T	CISD2	0.05	0.01	4.66E-06	-0.01	0.02	0.4071	0.03	0.01	4.42E-04
	4	186,514,078	rs28567706	C	G	SORBS2	0.49	0.09	4.05E-08	0.08	0.07	0.2745	0.23	0.05	2.22E-05
	8	42,527,386	rs11785369	A	C	CHRNA3	0.09	0.02	8.83E-08	0.06	0.03	2.46E-02	0.08	0.01	7.88E-09
	9	21,673,859	rs184042824	C	T	MIR31HG	-0.31	0.06	5.70E-08	-0.03	0.09	0.7136	-0.23	0.05	1.53E-06
	11	97,713,579	rs117029742	T	C	BG182718	-0.30	0.05	1.02E-08	0.07	0.07	0.3574	-0.17	0.04	5.28E-05
	14	44,608,436	rs10133756	C	G	AA333164	-0.31	0.05	4.11E-09	0.04	0.07	0.5277	-0.18	0.04	1.26E-05
	15	78,882,925	rs16969968	G	A	CHRNA5	-0.06	0.01	6.21E-09	-0.10	0.02	2.84E-10	-0.07	0.01	1.05E-17
	17	51,940,280	rs2877510	C	T	KIF2B	0.06	0.01	6.36E-06	0.01	0.02	0.5735	0.04	0.01	4.45E-05
	20	15,901,883	rs6135601	G	T	MACROD2	-0.13	0.03	5.53E-06	0.02	0.04	0.5457	-0.08	0.02	8.18E-04
	22	42,058,846	rs132786	G	A	XRCC6	-0.07	0.01	4.07E-06	0.02	0.02	0.2141	-0.03	0.01	5.29E-03

EA, effect allele; NEA, Non-effect allele; EAF, effect allele frequency; BP, base-pair position according to GRCh37/hg19; FTND, Fagerström Test for Nicotine Dependence; TTFC, time to first cigarette.

Table 3. Genes/loci showing convergent association between FTND and TTFC

Marker Info						FTND			TTFC			GENE
CHR	BP	SNP	EA	NEA	EAF	BETA	SE	P	BETA	SE	P	
2	4,073,151	rs13425218	G	C	0.68	-0.13	0.03	3.80E-05	-0.06	0.01	2.11E-05	DA409732
2	137,983,050	rs17269864	C	A	0.67	-0.10	0.02	2.77E-06	-0.04	0.01	3.17E-05	THSD7B *
2	185,787,483	rs56321552	G	C	0.95	0.24	0.06	2.20E-05	NL	NL	NL	ZNF804A **
2	185,639,136	rs78011041	T	G	0.97	NL	NL	NL	0.12	0.03	3.42E-05	ZNF804A **
2	221,852,642	rs6709809	G	A	0.96	-0.24	0.06	4.18E-05	-0.13	0.03	2.59E-06	-
5	30,863,015	rs4502841	G	A	0.48	-0.09	0.02	1.46E-05	-0.05	0.01	2.14E-06	-
5	92,486,203	rs4526087	C	G	0.90	0.15	0.03	1.15E-05	NL	NL	NL	-
5	92,554,821	rs193049029	T	C	0.98	NL	NL	NL	-0.24	0.06	2.78E-05	-
8	42,527,386	rs11785369	A	C	0.90	0.14	0.03	2.28E-05	0.09	0.02	8.83E-08	CHRNA3-CHRNA6
9	136,789,062	rs7847503	A	G	0.97	-0.23	0.06	4.32E-05	NL	NL	NL	VAV2
9	136,768,563	rs10993850	C	A	0.91	NL	NL	NL	0.08	0.02	3.93E-05	VAV2
11	72,301,503	rs341047	G	A	0.88	-0.14	0.03	3.07E-05	-0.06	0.02	3.83E-05	PDE2A
11	97,713,579	rs117029742	T	C	0.98	-0.64	0.12	1.04E-07	-0.30	0.05	1.02E-08	BG182718
15	78,882,925	rs16969968	G	A	0.64	-0.21	0.02	3.96E-19	-0.06	0.01	6.21E-09	CHRNA5-CHRNA3-CHRNA4
16	6,124,714	rs9927211	G	C	0.23	-0.13	0.03	1.83E-05	NL	NL	NL	RBFOX1 ***
16	6,071,910	rs12444143	T	C	0.40	NL	NL	NL	-0.05	0.01	3.56E-05	RBFOX1 ***
17	51,926,097	rs3966061	C	T	0.17	0.12	0.03	2.14E-05	0.06	0.01	1.46E-05	KIF2B
20	61,989,658	rs45623037	G	C	0.92	-0.21	0.05	2.75E-05	NL	NL	NL	CHRNA4
20	60,522,424	rs4925328	T	G	0.43	NL	NL	NL	0.05	0.01	4.78E-05	CHRNA4
22	36,503,250	rs16996373	G	T	0.84	-0.12	0.03	3.18E-05	-0.06	0.01	8.93E-06	APOL3

EA, effect allele; NEA, Non-effect allele; EAF, effect allele frequency; BP, base-pair position according to GRCh37/hg19; FTND, Fagerström Test for Nicotine Dependence; TTFC, time to first cigarette; NL, not listed because the P-value > 5x10⁻⁵; *, linked to alcohol addiction;⁴³ **, linked to schizophrenia;^{58,59} ***, linked to cocaine addiction.⁴² Genes relevant to addictions or neuropsychiatric co-morbidities are highlighted in bold.

Table 4. Summary of gene-association results

Gene Information			FTND		TTFC		Functions *
Name	Chr	Position	P value	FDR q value	P value	FDR q value	
RPE65	1	68894506	0.49513	1.0000	0.00004	0.0673	congenital amaurosis
CHD1L	1	146714290	0.04676	0.7808	0.00003	0.0636	cancer gene
TTFCP2L1	2	121974163	0.89730	1.0000	0.00001	0.0279	pluripotency, development
COPB2	3	139076432	0.00001	0.0229	0.64899	1.0000	lung cancer
LOC102723704	4	103698193	0.08404	0.8275	0.00004	0.0673	ncRNA
UBE2D3	4	103715539	0.12397	0.8807	0.00005	0.0674	ubiquitination
LOC105377348	4	103750470	0.32928	1.0000	0.00006	0.0676	ncRNA
CISD2	4	103790134	0.36281	1.0000	0.00004	0.0673	intracellular calcium homeostasis
SLC9B1	4	103822084	0.51668	1.0000	0.00004	0.0673	sodium/hydrogen exchanger
DNAH8	6	38683116	0.16941	0.9251	0.00006	0.0676	sperm and respiratory cilia motility
AGR3	7	16899029	0.37663	1.0000	0.00008	0.0827	cancer gene
RELN	7	103112230	0.31646	1.0000	4.47E-06	0.0223	schizophrenia, autism, cell adhesion
CHRNA3	8	42552561	0.03471	0.7313	0.00005	0.0674	nicotinic receptor
MIR31HG	9	21454266	0.03006	0.7241	0.00005	0.0676	lncRNA, cancer regulation
LOC389765	9	88420916	0.06893	0.8117	0.00006	0.0698	-
TMEM132B	12	126106997	0.19739	0.9368	0.00009	0.0847	intracranial aneurysm
SLC46A3	13	29274217	0.54791	1.0000	0.00004	0.0673	Lysosome/cytoplasm transport
KLHL1	13	70274724	0.00271	0.4838	0.00002	0.0579	calcium signaling, cancer
IREB2	15	78730517	9.55E-11	4.76E-07	0.00014	0.1254	lung diseases
HYKK	15	78799905	3.59E-17	8.93E-13	1.20E-07	9.92E-04	lung diseases
PSMA4	15	78832746	7.80E-11	4.76E-07	1.76E-05	5.48E-02	lung diseases
CHRNA5	15	78857861	1.53E-15	1.27E-11	9.51E-08	9.92E-04	nicotinic receptor
CHRNA3	15	78887646	1.00E-15	1.25E-11	6.26E-08	9.92E-04	nicotinic receptor
CHRNA4	15	78916635	1.47E-10	6.12E-07	2.60E-06	1.62E-02	nicotinic receptor
INO80C	18	33048290	0.07345	0.8117	1.01E-05	0.0359	chromatin remodeling/silencing
SSTR4	20	23016056	0.23580	0.9667	2.32E-05	0.0579	somatostatin receptors
CHRNA4	20	61974661	1.39E-06	0.0049	0.0013	0.3773	nicotinic receptor
XRCC6	22	42017345	0.09636	0.8438	0.0001	0.0724	cancer gene
NHP2L1	22	42069936	0.30160	0.9951	0.0001	0.0835	chromatin remodeling/silencing
MEI1	22	42095517	0.42723	1.0000	0.0001	0.0676	meiotic chromosome synapsis

FTND, Fagerström Test for Nicotine Dependence; TTFC, time to first cigarette; *, functions relevant to smoking or neuropsychiatric co-morbidities are highlighted in bold. FDR q-values below 0.05 are highlighted in bold.

Table 5. Summary of pathway enrichment analyses

Pathway	Genes Found in the Pathway	# Gene in Pathway	# Gene Observed	# Gene Expected	Observed /Expected Ratio	Raw P-Value	Adjusted P-Value
Neuroactive ligand-receptor interaction	CHRNA3,CHRNA4,CHRNA5,CHRNA6,CHRNB3,CHRNB4,GABRG3,GRM5,SSTR1,SSTR4,THRB	272	11	1.65	6.66	1.03E-06	5.56E-05
Endocytosis	AP2A2,EHD3,FGFR2,IQSEC1,MET,RAB5C	201	6	1.22	4.91	0.0015	0.0405
Intestinal immune network for IgA production	HLA-DMB,HLA-DRB5,IL15	48	3	0.29	10.29	0.0031	0.0540
Lysosome	AP1S3,LIPA,MANBA,SUMF1	121	4	0.74	5.44	0.0065	0.0694
Protein export	SPCS3,SRP9	23	2	0.14	14.31	0.0086	0.0694
Cell adhesion molecules (CAMs)	CDH4,HLA-DMB,HLA-DRB5,NRCAM	133	4	0.81	4.95	0.0090	0.0694
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	CACNA1D,CACNG4,CTNNA3	74	3	0.45	6.67	0.0105	0.0709
Dilated cardiomyopathy	ADCY4,CACNA1D,CACNG4	90	3	0.55	5.49	0.0177	0.0816
Purine metabolism	ADCY4,PAPSS2,PDE2A,PDE4B	162	4	0.98	4.06	0.0174	0.0816
Huntington's disease	AP2A2,GRM5,PPARG,TAF4	183	4	1.11	3.60	0.0259	0.0816
MAPK signaling pathway	CACNA1D,CACNG4,DUSP16,FGFR2,MRAS	268	5	1.63	3.07	0.0244	0.0816
Melanogenesis	ADCY4,FZD8,WNT16	101	3	0.61	4.89	0.0239	0.0816
Allograft rejection	HLA-DMB,HLA-DRB5	37	2	0.22	8.90	0.0213	0.0816
Type I diabetes mellitus	HLA-DMB,HLA-DRB5	43	2	0.26	7.66	0.0282	0.0816
Asthma	HLA-DMB,HLA-DRB5	30	2	0.18	10.97	0.0143	0.0816
Graft-versus-host disease	HLA-DMB,HLA-DRB5	41	2	0.25	8.03	0.0258	0.0816
Rheumatoid arthritis	HLA-DMB,HLA-DRB5,IL15	91	3	0.55	5.43	0.0182	0.0816
Chemokine signaling pathway	ADCY4,TIAM2,VAV2,XCL1	189	4	1.15	3.48	0.0287	0.0816
Regulation of actin cytoskeleton	FGFR2,MRAS,TIAM2,VAV2	213	4	1.29	3.09	0.0416	0.0944
Autoimmune thyroid disease	HLA-DMB,HLA-DRB5	52	2	0.32	6.33	0.0400	0.0944
Hedgehog signaling pathway	RAB23,WNT16	56	2	0.34	5.88	0.0457	0.0944
Basal cell carcinoma	FZD8,WNT16	55	2	0.33	5.99	0.0442	0.0944
Tight junction	CTNNA3,EPB41L1,MRAS	132	3	0.8	3.74	0.0469	0.0944
Inositol phosphate metabolism	IMPA1,MINPP1	57	2	0.35	5.78	0.0472	0.0944
Axon guidance	MET,NTNG1,ROBO2	129	3	0.78	3.83	0.0443	0.0944
Staphylococcus aureus infection	HLA-DMB,HLA-DRB5	55	2	0.33	5.99	0.0442	0.0944

Statistically significant adjusted p-values are highlighted in bold.